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Effect of tuftsin and oligotuftsins on chemotaxis and chemotactic selection in *Tetrahymena pyriformis*

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Abstract

The chemotactic properties of tuftsin (H-TKPR-OH), tuftsin derivatives (H-KPR-OH, H-TKPKG-NH_2, Ac-TKPKG-NH_2) and TKPKG-based oligotuftsins (T20, T30, T40) were investigated in *Tetrahymena pyriformis* GL. In contrast to its effects on Mammalia, tuftsin elicited chemorepellent or neutral responses; truncation of the N-terminal part (KPR) led to similar results, though with more neutral effects. The significance of the C-terminal part of the molecule was revealed by the chemoattractant properties of TKPKG, which are nevertheless abolished by acylation. Among the oligotuftsins, T20 and T40 were chemoattractants at higher concentrations (10^{-9}–10^{-6} M), while T30 had a wide-ranging chemorepellent effect, indicating that chemotaxis is elicited in *Tetrahymena* only by ligands with optimal physicochemical characters (mass, conformation, etc.). The chemotactic selection data indicated that tuftsin-induced chemotaxis results from fairly short-term signalling in *Tetrahymena*.

Keywords: Tuftsin; Oligotuftsin; Chemotaxis; Selection; *Tetrahymena*

1. Introduction

Tuftsin (TKPR) is a hormone-like tetrapeptide derived from the Fc heavy chain of IgG by a two-step enzymatic cleavage. The tetrapeptide sequence results from maturation by proteases in the spleen and neutrophil membranes (Najjar et al., 1968). Studies on the conformation of the molecule are rather controversial; a β-turn (Sucharda-Sobczyk et al., 1979), and intramolecular interaction between the lysine ϵ-NH_2-group and the C-terminal COOH group (Nikiforovich, 1978), have both been proposed. Receptor kinetic studies have revealed that the active form of the molecule is bound by tuftsin receptors comprising two subunits (Bump et al., 1987). Significant numbers of these receptors (50–100,000/cell) are expressed in vertebrate neutrophils (Nair et al., 1978), the monocyte/macrophage pool and lymphocytes (Nishioka et al., 1981); erythrocytes have no significant tuftsin binding capacity (Stabinsky et al., 1978). Induction of phagocytosis is one of the commonest cell-physiological effects of tuftsin (Coleman, 1986), accompanied by the production of killing components such as reactive oxygen species.

The chemotactic responsiveness of neutrophils and mononuclear cells is also enhanced by tuftsin (Babcock et al., 1983). However, some reports indicate that this ligand has inhibitory effects (Pohajdak et al., 1986). There is evidence that tuftsin interacts with other chemotaxis receptors; for instance, it inhibits cell migration towards fMLP or IL-8 gradients (Wiedermann et al., 1991). It alters cyclic nucleotide levels in impaired inflammatory cells, restoring their chemotactic responsiveness (Roch-Arveiller et al., 1982). Tuftsin and its analogues have stimulating and potentiating effects on monocyte chemotaxis, which is impaired in Hodgkin (Lukacs et al., 1983) and SLE (Lukacs et al., 1984) patients, indicating that these ligands promote phagocytosis by modulating earlier signalling steps.

Oligotuftsins are non-toxic TKPR-derived oligopeptides (Mező et al., 2001) that enhance immune responses (Mező

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et al., 2001). They are pharmacologically significant in that active substances can be conjugated to the lysine residue ϵ-amino-groups (Mező et al., 2003). Oligotuftsins comprising various numbers of TKPKG repeat units exert cell-type dependent chemotactic effects on monocyte cell lines though not on fibroblasts (Mező et al., 2004).

The wide-ranging distribution of the tuftsin sequence in biomolecules from many different taxa enables us to investigate the functional significance of tuftsin in a relatively primitive eukaryote. The model cell used in the present paper is the unicellular eukaryotic ciliate *Tetrahymena pyriformis*, which has been well studied by cell biologists (Csaba, 1985). Tuftsin stimulates phagocytosis of *Tetrahymena*, but the KPR derivative, a tuftsin inhibitor in Mammalia, elicited a higher phagocytic response in this protozoon (Csaba et al., 1986). The highly selective chemotactic responsiveness to slight alterations in relatively small and large ligands including amino acids (Kőhidai, 1999), insulin derivatives (Csaba et al., 1994) and bradykinin analogues (Kőhidai et al., 2002) make *Tetrahymena* suitable for evaluating new, potentially chemoattractant substances. A selection technique (Kőhidai and Csaba, 1998) based on the selection of chemotactically positive responder cells and exploiting the short cell cycle of our model organism allows us to distinguish between long- and short-term chemotactic receptor-dependent responses.

The main objectives of our work were to determine:

(i) whether tuftsin and oligotuftsin analogues have chemotactic properties in a unicellular eukaryotic model;
(ii) whether the presumed chemotactic behaviour induced by oligotuftsins involves a long- or short-term receptor pool in *Tetrahymena*.

2. Materials and methods

2.1. Cells and culturing

Tetrahymena pyriformis GL cells were maintained in axenic cultures containing 1% tryptone and 0.1% yeast extract (Difco, Michigan, USA). Logarithmic growth phase cell cultures (48 h) were assayed at a density of 10^4 cells/ml.

2.2. Synthesis of ligands

H-[Thr-Lys-Pro-Lys-Gly]$_n$-NH_2 ($n = 1$, 2 [T10], 4 [T20], 6 [T30] or 8 [T40]) were prepared manually by stepwise SPPS on MBHA resin (Nova Biochem) with a capacity of 1.1 mmol/g, while tuftsin and its N-terminally truncated derivative KPR was synthesized on PAM resin ($c = 0.67$ mmol/g). A Boc/Bzl strategy was applied during synthesis using the following amino acid derivatives: Boc-Gly-OH, Boc-Pro-OH, Boc-Lys(ClZ)-OH and Boc-Thr(Bzl)-OH (all from Reanal, Budapest, Hungary). The synthetic protocol was: (i) resin wash with DCM (3 × 0.5 min); (ii) cleavage of Boc group with 33%TFA/DCM (2 × 20 min); (iii) resin wash with DCM (5 × 0.5 min); (iv) neutralization with 10% DIEA/DCM (4 × 1 min); (v) resin wash with DCM (4 × 0.5 min); (vi) coupling with DCC/HOBt-activated Boc-amino acid derivatives (3 equivalents of each to the capacity of the resin) for 60 min; (vii) resin wash with DMF (2 × 0.5 min) then with DCM (3 × 0.5 min); (viii) monitoring by ninhydrin assay (Kaiser et al., 1970) or isatin test (Kaiser et al., 1980). Anhydrous hydrogen fluoride (10 ml in the presence of 0.5 g *p*-cresol) was used to remove the side chain protecting groups and to cleave the desired peptide from the resin. All chemicals were Fluka products (Buchs, Switzerland), while the solvents were purchased from Reanal.

2.3. Reverse phase high performance liquid chromatography (RP-HPLC)

Analytical RP-HPLC was performed on a Waters (Nihon Waters Ltd., Tokyo, Japan) HPLC system using a Phenomex Jupiter C_{18} column (250 × 4.6 mm I.D.) with 5 μm silica (300 Å pore size) (Torrance, CA) as a stationary phase. Linear gradient elution (0 min 0% B; 5 min 0% B; 50 min 90% B) with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile–water (80:20, V/V)) was used at a flow rate of 1 ml/min at ambient temperature. Absorbance peaks were detected at 214, 254 and 280 nm. The samples were dissolved in eluent A. The crude products were purified on a semipreparative Phenomex Jupiter C_{18} column (250 × 10 mm I.D.) with 10 μm silica (300 Å pore size) (Torrance, CA). The flow rate was 4 ml/min. The same eluents were applied with a linear gradient from 0% B to 30% B in 30 min.

2.4. Amino acid analysis

The amino acid compositions of the peptides were determined using a Beckman Model 6300 amino acid analyser (Fullerton, CA, USA). Prior to analysis the samples were hydrolysed in 6 M HCl in sealed evacuated tubes at 110 °C for 24 h.

2.5. Mass spectrometry

Positive ion electrospray ionization mass spectrometric (ESI-MS) analyses were performed on a PE API 2000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada). The spray voltage was set to 4.8 kV and a 30 V orifice voltage was used. Samples (5 μl) were dissolved in methanol–water (1:1 V/V) containing 0.1% acetic acid and injected with a flow rate of 100 μl/min. The instrument was used in Q_1 scan mode in the range *m/z* 400–1700, with a step size of 0.3 amu and a dwell time of 0.5 ms.

2.6. Chemotaxis assay

The chemotactic responsiveness of *Tetrahymena* cells was evaluated by the two-chamber capillary assay described previously (Kőhidai et al., 1995). The tips of a multi-8-channel micropipette served as inner chambers and were filled with different concentrations of the tuftsin or oligotuftsin derivatives (10^{-12}–10^{-6} M), while the wells of a plastic plate served as outer chambers and were filled with cell cultures. During concurrent runs, pure culture medium served as control. Control samples were evaluated in parallel in each case to eliminate any disturbances consequent on spontaneous mutations. The incubation time was 20 min; its relative brevity facilitated the measurement of purely gradient-directed chemotactic responses and prevented contamination from randomly-running chemokinetic responder cells (Kőhidai et al., 2000). The samples were fixed in 4% formaldehyde containing PBS and the number of positively-responding cells was counted occulometrically using a Neubauer hemocytometer.

2.7. Chemotactic selection

This technique reveals the capacity of a signal molecule to form subpopulations from a mixed cell culture by differential chemotaxis. First, we applied the chemotaxis assay described above. At the end of incubation, the positive responder cells were transferred to fresh culture medium for cultivation. Cultures selected with a tuftsin derivative (T) and control cultures (C) were consecutively transferred every 48 h. The chemotactic responses were determined in the following combinations: T/T, cells selected with the tuftsin in the first run and assayed with the same signal substance in the second run; T/C, cells selected with the tuftsin in the first run and assayed with the control in the second run; C/T, cells selected with the control in the first run and assayed with the signal

in the second run; C/C, cells selected with the control in the first run and assayed with the control in the second run.

2.8. Statistical evaluation

Sixteen experiments evaluating the effects of each tuftsin and oligotuftsin were performed in parallel. ANOVA (Origin 4.0) was used to evaluate statistical significances. In the figures the following marks refer to the significant differences: *x*, $p < 0.05$; *y*, $p < 0.01$; *z*, $p < 0.001$.

3. Results and discussion

3.1. Chemotaxis induced by tuftsin and short derivatives

In the first series of experiments our purpose was to clarify whether human tuftsin (H-TKPR-OH) and its short peptide derivatives (H-KPR-OH, H-TKPKG-NH_2, Ac-TKPKG-NH_2) are chemoattractive in *Tetrahymena*. Data obtained with different human tuftsin concentrations (Fig. 1a) show that it has no chemoattractant activity in this organism, despite similarities in phagocytotic potency, and a significant chemorepellent activity was detected in the 10^{-9}–10^{-8} M range. The tripeptide H-KPR-OH, designed as a tuftsin inhibitor for higher phylogenetic level organisms, shows almost the same profile, though its activity is neutral rather than chemorepellent, suggesting that the C-terminal Arg is important in chemorepellent potency.

The sensitivity of chemotactic signalling in *Tetrahymena* was evaluated by another peptide (H-TKPKG-NH_2, based on dog tuftsin [H-TKPK-OH], the building block of the oligotuftsins tested) and its acetylated derivative (Fig. 1b). Replacement of the C-terminal Arg of human tuftsin with the Lys-Gly sequence results in significant chemoattraction over a wide concentration range (10^{-9}–10^{-6} M), and positive though non-significant chemotactic effects even at the lowest concentration tested (10^{-12} M). The significance of C-terminus in biological activity has been described previously. The C terminal Pro-Arg sequence appears to be responsible for lymphokine secretory potential (Paegelow and Werner, 1986). On the basis of our present data, removal of the large Arg with high accessible surface area (93.8) (Rose et al., 1985) and substitution of the small Gly with a correspondingly low accessible surface area (25.2) seems to enhance efficacy. These observations correspond closely with our previous studies in which the terminal parts of several chemotactic ligands—dipeptides (Kőhidai et al., 1997), bradykinin (Kőhidai et al., 2002) and EWS-motif-containing peptides (Kőhidai et al., 2003a,b)—were found to be significant.

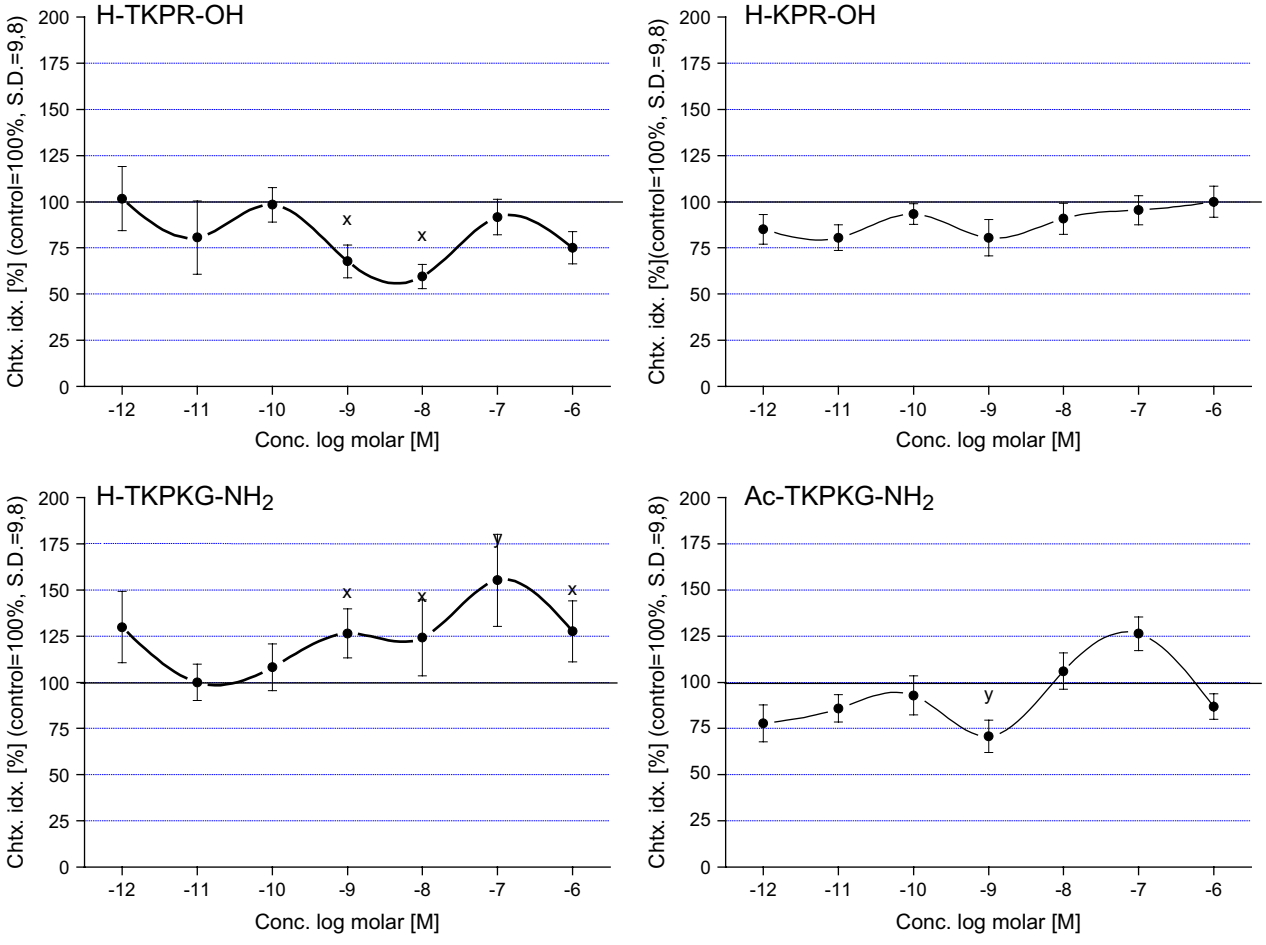

Fig. 1. Concentration course study showing chemotactic effects of human tuftsin and its derivatives (H-KPR-OH, H-TKPKG-NH_2 and Ac-TKPKG-NH_2) on *Tetrahymena pyriformis* GL. *x*, $p < 0.05$; *y*, $p < 0.01$; *z*, $p < 0.001$.

Cyclization of tuftsin in the apolar biophase of a receptor, with a salt bridge between the lysine ϵ-amino group and the arginine carboxyl group, may be important for the biological effects of the ligand (Chipens et al., 1981), so we should also consider the physico-chemical conditions of the Lys residue. The other modification we examined, acetylation of TKPKG, results in the loss of chemoattractant character. This derivative has a neutral profile over a wide concentration range (10^{-12}–10^{-10} M), interrupted by two weak, non-significant and opposite effects (10^{-9} M, chemorepellent; 10^{-7} M, chemoattractant), indicating a close dependence of function on structure in the N-terminal as well as the C-terminal part of the molecule.

3.2. Chemotactic behaviour of oligotuftsins

In the second panel of experiments, the chemotactic effects of the oligotuftsins T20, T30 and T40 were analysed (Fig. 2). T20 and T40, containing 4 and 8 TKPKG units respectively, gave two-peaked profiles of concentration dependence closely homologous to the TKPKG monomer, with a characteristic chemoattractant effect at 10^{-12} M and a broad second peak at 10^{-9}–10^{-7} M. The functional similarity between these 20 and 40 residue ligands and the pentapeptide is informative, because neither theoretical nor conformation-fitting algorithms have found representative conformations for these oligopeptides. Presumably the larger ligands do not have complicated three-dimensional structures, since the reacting residues appear to be no less available to the chemotactic receptors than they are in the monomer. Comparison of these data with those obtained using rodent J774 monocytes and MRC5 fibroblasts indicates that the unicellular model is more sensitive, while the vertebrate cells respond over wide concentration range and exhibit weak positive (monocyte) or negative (fibroblast) chemotaxis (Mező et al., 2004).

T30, containing 6 TKPKG units, elicited a chemorepellent response at each concentration tested, with significant effects at 10^{-10} and 10^{-9} M. It is possible that T30 has a more flexible conformation than the other oligomers, and some residues essential for chemotaxis are rendered cryptic. Verification of this hypothesis requires further investigation, particularly of the relationship between the conformations of T30 and T40. The greater sensitivity of *Tetrahymena* compared to vertebrate models is also interesting here, as the aforementioned vertebrate cells responded to T30 as they did to T20 and T40, whilst *Tetrahymena* responded differently to the presumably slight structural alteration.

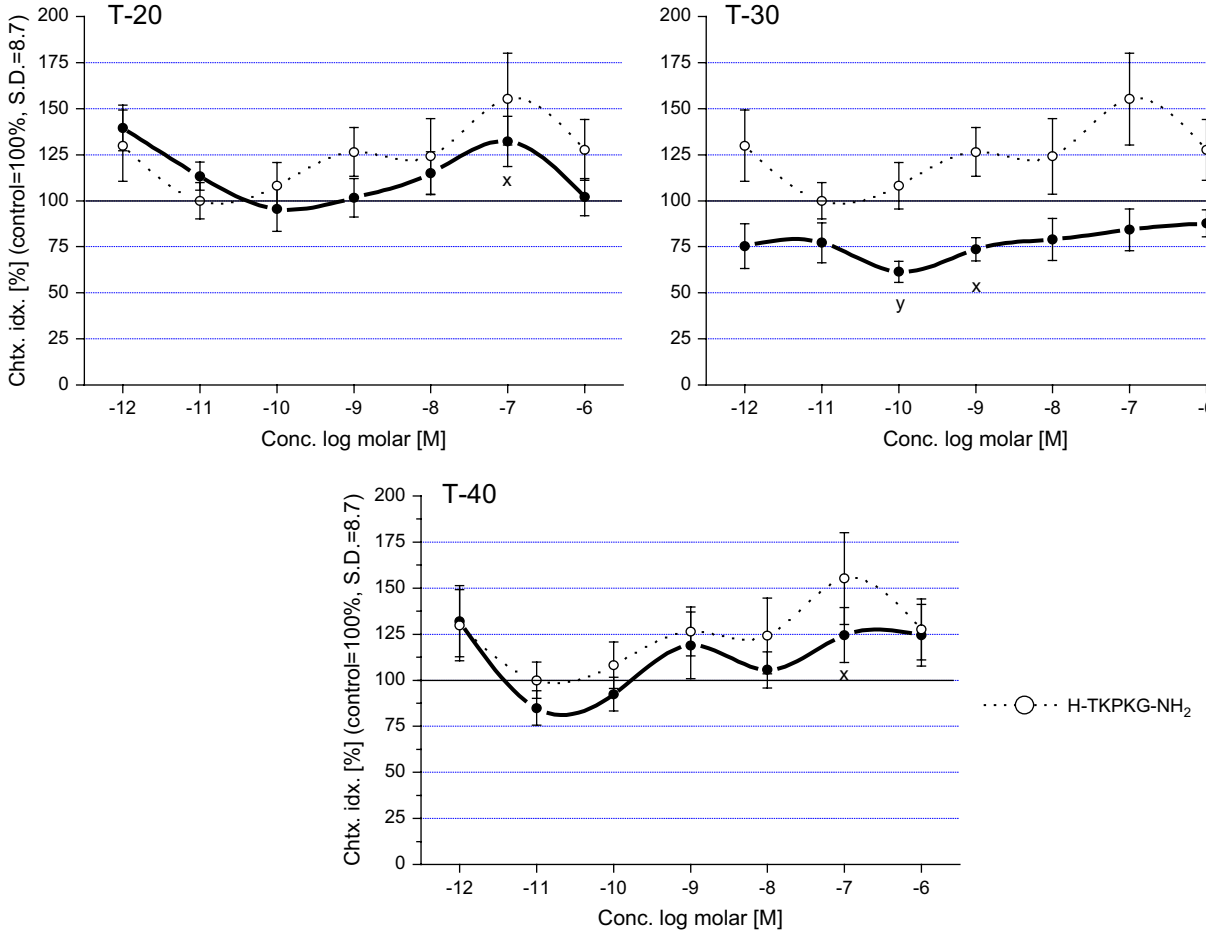

Fig. 2. Concentration course study showing chemotactic effects of oligotuftsins comprising 4 (T20), 6 (T30) or 8 (T40) TKPKG sequence units on *Tetrahymena pyriformis* GL. The dotted line represents the chemotactic potential of the basic TKPKG sequence. *x*, $p < 0.05$; *y*, $p < 0.01$; *z*, $p < 0.001$.

3.3. Ability of tuftsin derivatives and oligotuftsins to select subpopulations via chemotaxis

Finally, chemotactic selections were analysed. Since human tuftsin, tuftsin inhibitor and T30 oligotuftsin had all proved chemorepellent, only the ligands TKPKG, T20 and T40 were investigated in these experiments (Fig. 3). TKPKG induced a significantly higher chemotactic response in the selected sub-populations (TKPKG/TKPKG) than the control (TKPKG/C). The response elicited by TKPKG is significantly stronger in selected sub-populations than in mixed ones (C/TKPKG), indicating that selection increases the number of functional chemotactic receptors present. Presumably the increased chemotactic responsiveness of the selected cells involves long-term chemotactic receptors, since the cultures were assayed again after 7 days of selection, when the responding cells represent about the 70th generation of the selected *Tetrahymena* cells. This long-term, enhanced chemotactic responsiveness to a tuftsin derivative seems to be unique, or at least distinctive, because the receptors responsible for phagocytosis do not remain active for long after induction (Csaba et al., 1986). However, chemotaxis is considered as a target reaction of phagocytosis, so the contrast suggests that there are significant differences in the components or dynamics of the signalling mechanisms involved.

T20 and T40 did not elicit enhanced chemotactic responsiveness in the offspring generations. The chemotactic responsiveness of the sub-populations selected with culture medium differed from the results of the concentration course study; it was depressed in both cases (C/T20 and C/T40). A possible explanation for this effect is that the control medium contains organic components (tryptone and yeast extract). Inorganic media such as Losina-Losinsky solution (Losina-Losinsky, 1931) lack components that might interfere with chemotactic selection. However, such media are non-physiological and unsuitable for chemotaxis, so we chose fresh medium as our control.

The sub-populations selected with T20 show a chemotactic responsiveness parallel to those selected with control medium (T20/C) or T20 (T20/T20). This suggests that selection with T20 oligotuftsin, as in the case of other bioactive substances such as RANTES (Kőhidai and Csaba, 1998), endothelin-1 (Kőhidai et al., 2001) and progesterone (Kőhidai et al., 2003a,b) and in contrast to the TKPKG monomer, has no long-term effect; these ligands work as ‘‘acute’’, short-term chemoattractants. T40 exhibited a similar short-term responsiveness, but the relative ratios of T40 and control selected populations (T40/T40/T40/C vs. C/T40/C/C) indicates the potential for long-term chemotactic responsiveness ($Chem_{sel}$ = 1.66).

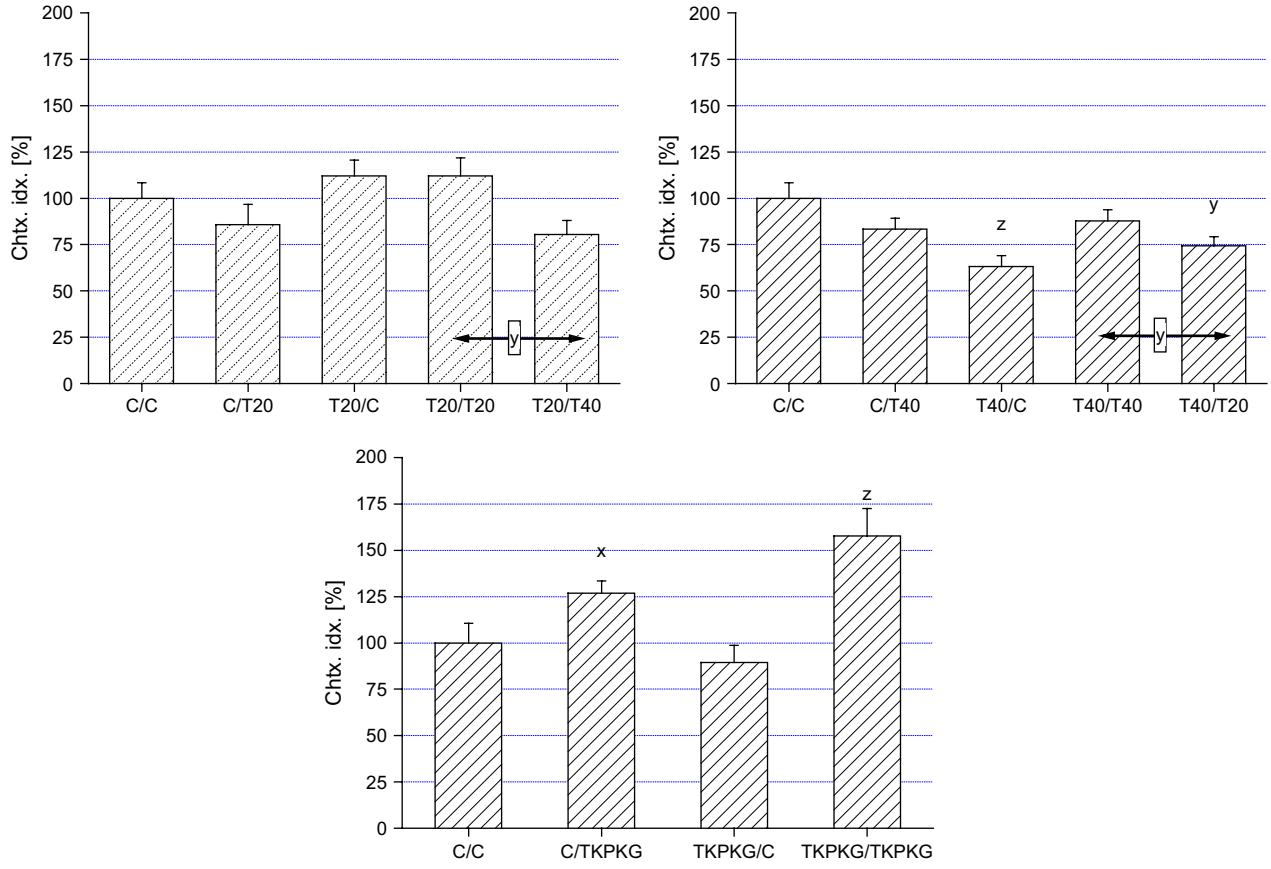

Fig. 3. Effect of chemotactic selection on the chemotactic responsiveness of *Tetrahymena* cells selected by T20 and T40 oligotuftsins or the TKPKG tuftsin derivative. *x*, $p < 0.05$; *y*, $p < 0.01$; *z*, $p < 0.001$; arrows indicate levels of significance in study of cross-reactivity between the two chemoattractant oligotuftsins.

Cross-reactivity between the two oligotuftsins was also evaluated. The homologous tuftsin derivatives (T20/T20 or T40/T40) elicited greater chemoattractive responsiveness than the heterogeneous (T20/T40 or T40/T20) ones. In contrast to the short-term selection described above, this selectivity should be considered distinctive, inasmuch as structurally closely-related ligands are distinguished by the selected *Tetrahymena* cells.

More than one chemotactic receptor capable of signalling in tuftsin-mediated chemotaxis may be expressed. Therefore, the chemotactic behaviour of our model cell might also distinguish the appropriate derivatives of other ligands (bradykinin and endothelin derivatives) with two or more known membrane receptors (Kőhidai et al., 2001, 2002).

4. Conclusions

The data obtained from our present investigations indicate that tuftsin and its derivatives act as potent chemoattractant and chemorepellent ligands in unicellular eukaryotes. The chemotactic responsiveness of *Tetrahymena* shows that even slight structural modifications of tuftsin (e.g. H-TKPR-OH vs. H-TKPKG-NH_2 vs. Ac-TKPKG-NH_2) are detected by the cells. These sensitive chemotactic receptors discriminate not only relatively small ligands, but perhaps also—as the oligotuftsin results showed—larger peptide ligands.

The chemotactic selection with oligotuftsins and TKPKG indicates that the chemotactic receptors involved belong to the short-term persisting category, but the long-term positive selection with TKPKG raises the possibility that more than one chemotactic receptor (subtype?) is involved in tuftsin-induced chemotaxis in *Tetrahymena*.

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